IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: SAMAIN, Eric et al.

Title: METHOD FOR PRODUCING OLIGOPOLYSACCHARIDES

Appl. No.: 10/019,954

Filing Date: 5/24/2002

Examiner: Rebecca E. Prouty

Art Unit: 1652

Conf. No.: 6242

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents

Sir:

I, Eric Samain, declare as follows:

1. I am one of the inventors of the captioned application.

2. I am currently employed at Centre National de la Recherche Scientifique—Centre de Recherches sur les Macromolécules Végétales ("CNRS-CERMAV").

3. My academic background and work experience are summarized in my *curriculum vitae*, which is attached as Exhibit A. Briefly, I did my PhD on the microbiology of methanogenic fermentation in a laboratory of the French National Institute for Agronomical Research (I.N.R.A.) located in Lille, France. I was recruited as a research engineer in 1982 in the same laboratory to pursue my PhD work on the physiology of bacteria

involved in methanogenesis. I then worked on the bacterial production of glycosyl-hydrolases for agricultural uses and developed a patented fermentation process for the high yield production of a thermophilic xylanase. In 1990 I was offered a position at the CNRS (National Center for Scientific research) in Grenoble to develop new fermentation processes related to the degradation and the biosynthesis of carbohydrate in a CNRS institute (CERMAV) which is considered as the most important European Research Institute devoted to the study of carbohydrates. Since 1995, my activities have been focused on the synthesis of oligosaccharides by metabolically engineered bacteria, and my research in this field has been recognized with the esteemed "Cristal of CNRS" awards in 2004, and it has earned me invitations to lecture at several international meeting. Over the years, I have produced more than 45 research papers in microbiology and biotechnology and two book chapters on microbial oligosaccharide production. I have submitted 4 patents in the area of oligosaccharide synthesis and have directed the research of several Ph.D. students and Master's students.

4. I have read and understand the Office Actions dated December 29, 2005, and January 9, 2008. Among other rejections, I understand that the 2008 Office Action rejects the claims as obvious over the following references, either alone or in combination:

(a) ("Koizumi et al.") Koizumi S., Endo T., Tabata K. and Ozaki A. (1998) Large scale production of UDP-galactopse and globotriose by coupling metabolically engineered bacteria, Nature Biotechnolo. 16, 847-850

(b) ("Bettler et al.") Bettler E., Samain E., Chazalet V., Bosso C., Heyraud A., Joziasse D.H., Wakarchuk W.W., Imberty A., Geremia R.A. (1999) The living factory: in

vivo production of N-acetyllactosamine containing carbohydrates in E. coli., Glycoconj. 6(3):205-12.

(c) ("Dykhuizen et al.") Dykhuizen D., Hartl D. (1978) Transport by the lactose permease of Escherichia coli as the basis of Lactose killing, Journal of Bacteriology, 135, 876-882

(d) Ahmed S, Booth IR (1983) The effect of beta-galactosides on the proton motive force and growth of Escherichia coli, J Gen Microbiol. 129(8):2521-9.

5. The 2008 Office Action (page 4, lines 3-5) states that "claims 1, 5-7, 9-12, 27, 28, 39, 47 and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over Bettler et al. in view of Kozumi et al." The rejection is explained in the 2005 Office Action, which states that Bettler et al. "teach the intracellular production of the oligosaccharide Galβ-4(GlcNAcβ-4)$_4$GlcNAc using a LacZ- E. coli" (2005 Office Action, page 8) and that Kozumi et al. "teach the production of the trisaccharide globotriose from lactose using a permeabilized LacZ- E. coli" (2005 Office Action, page 9). The 2005 Office Action concludes (page 10) that "it would have been obvious to use the transformed LacZ- E. coli of Kozumi et al.without permeabilizing the membrane as taught by Bettler et al." and that "a skilled artisan would have been motivated to overexpress this gene [lactose permease] in the bacteria of Kozumi et al. as lactose is the precursor used by Kozumi et al." (2005 Office Action, page 10).

6. I submit that while Bettler et al. is part of the "March 1999 volume" of Glycoconjugate Journal, the print and release date of the "March 1999 volume" of Glycoconjgate Journal is actually **September 24, 1999**, as proved by the document attached as exhibit B from the Editor.

7. That means that the effective date on which Bettler et al. has been made accessible to the public is after September 24, 1999, and therefore **after the priority date of the present application, which is July 7, 1999**. As a result Bettler et al. cannot be considered prior art for the rejection of pending claims 1, 5-7, 9-12, 27, 28, 39, 47 and 48.

8. In response to the rejection of the claims under 35 U.S.C. 103(a) presented in §3 of the Office Action, we have stated in response to previous Office Actions that "one of skill would have no motivation to combine Koizumi et al. and Bettler et al., much less any expectation of success, because it was known in the art that rapid uptake of sugars by lactose permease disrupts membrane function . . . which results in growth inhibition and eventually cell death [i.e] 'lactose killing.'" Amendment filed February 28, 2007, page 14. Nonetheless, the 2008 Office Action states that "this is not persuasive because lactose killing as reported in [Dykhuizen et al.] is present in E. coli cells that have been growing on a limited supply of lactose when they were then provided with excess lactose but not in cells growing on other carbons sources when supplied with lactose". 2008 Office Action, page 4 (emphasis in original).

9. I agree with the fact that cells growing on other carbons sources are not affected by the lactose killing and this was clearly written (on page 877, lines 16-20) in Dykhuizen et al. However these E. coli cells growing on other carbon sources have of course no reason to be killed by lactose, because their lactose permease is not induced, since they have been grown in absence of lactose.

10. On the contrary as shown in table 2 of Dykhuizen et al., cultivation of E. coli cells on glucose or galactose in presence of IPTG (which is an inducer of the lactose permease) results in a strong lactose killing effect. The authors conclude (on page 878, column 2, line 11-17) that **" there is strong correlation between the amount of lactose permease and the amount of lactose killing"**.

11. Thus, I state that the interpretation of Dykhuizen et al. by the Examiner contradicts this quoted statement within Dykhuizen et al. itself.

12. The 2008 Office Action also states that "the amount of growth inhibition produced by lactose can be diminished by reducing the rate of import of lactose into the cells and the presence of glucose or glycerol in the culture during the second phase of cell growth would do just that as they are well know to repress the lactose promoter" (2008 Office Action, page 5).

13. It is true that glucose (but not glycerol) represses the lactose promoter by a mechanism called catabolic repression. However, in the invention as claimed, the second phase of cell growth is carried out in carbon-limiting condition to precisely prevent this catabolic repression and enable the full expression of the lactose permease, which is a necessary condition for a very efficient system of oligosaccharide synthesis. One should keep in mind that the interest of this invention is its very high productivity and that we have later succeeded in obtaining by the process as claimed the **production of complex oligosaccharides at a concentration of more than 25 g/l** (see publication Fierfort and Samain, J. of Biotechnology 134 (2008) 261-265, in exhibit C).

14. A skilled artisan in this field would not have anticipated such excellent results. On the contrary, the skilled artisan would have considered that there was no industrial interest in developing a process whose yield would be limited by the lactose input due to the lactose killing effect. Thus, a skilled artisan would not have contemplated using a system as defined in the claims.

15. In addition, the lactose promoter and other catabolically repressed promoter such as the arabinose promoter are largely used in common expression vector and in particular in almost all the expression vectors that were used in the examples of the claimed invention to overexpress the genes for glycosyltransferases and other enzyme involved in sugar nucleotide biosynthesis that are required for the synthesis of complex oligosaccharides. Therefore, one skilled in the art would not have considered the process as claimed since partial repression by catabolic repression of the lactose promoter would affect not only the expression of the lactose permease but also the expression of other genes involved in oligosaccharide synthesis.

16. The 2008 Office Action also states, "Furthermore, a skilled artisan would be aware that even a low growth rate of the cells during the second phase could still be sufficient to produce large amount of the desired product" (2008 Office Action, page 5).

17. It is true that many products are produced in condition of low growth rate and this is actually the case of the claimed invention. However, the synthesis of oligosaccharide is an energy demanding process which requires metabolically active cells able to efficiently produce all the precursors such as the sugar nucleotides. The main fear that a skilled artisan

could have about the lactose killing is thus not the problem of slow growth but more the problem of irremediable damage to the cells, which would affect their metabolically activity and their energetic yield.

18. The strain used in reference Dykhuizen et al. is LacZ+. This designation means that cells as in Dykhuizen et al. hydrolyze and catabolize lactose.

19. In the claimed invention, the strain are LacZ- and the lactose accumulates intracellularly at high concentration. A skilled artisan would have feared that this accumulation could be detrimental for the cells by dramatically increasing the intracellular osmotic pressure (turgor). This increase in turgor can cause cell death because of membrane rupture, and bacterial cells are known to adapt to severe turgor increase by opening stretch activated channel to let small molecule exit. As a small molecule lactose is likely to exit through the activated channel and to create an energy consuming futile cycle by being reinternalized by the lactose permease.

20. I was the first to report and demonstrate that it is possible to maintain, for several hours and in an excellent state of metabolic activity, a high cell density population of E. coli cells that contain a high intracellular concentration of lactose (a metabolically active cell being defined as a cell that is capable to maintain its cellular integrity and to fulfill all the physiological functions of a living cell, e.g., protein and other macromolecule synthesis, ATP generation, and active transport). This condition is a prerequisite for the claimed invention because a metabolically active cell can express a recombinant glycosyltransferase, recycle

sugar nucleotides, and therefore glycosylate intracellular lactose to obtain the desired oligosaccharide.

21. However, as the accumulation of any metabolite is susceptible to be toxic for a cell, and the lactose killing effect is a well-known phenomenon, this prerequisite would not have been obvious for a microbial physiologist. Consequently, a microbial physiologist would not have been motivated to develop a system of oligosaccharide synthesis from lactose by living E. coli, considering the fact that Koizumi et al.and Bettler et al. described efficient systems, and a skilled artisan would have been more motivated to improve either the Koizumi et al. or the Bettler et al. process.

22. I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: _____ By: _____
Eric Samain

EXHIBIT A

Curriculum Vitae

SAMAIN Eric
Date of birth March 27 1956 in KENITRA (Maroc)
Married , 3 children
Professional Address
CERMAV
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Phone 33 4 76 03 76 48
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Education

- 1979 : Master in Biochemistry at the University of Lille
- 1983 : PhD thesis at the University of Lille on the microbiology of the methanogenic fermentation

Post-doctoral fellowship

- 1985 in the Department of Biochemistry of the University of Georgia (Athens,USA) : purification and characterization of cytochromes from sulphate reducing bacteria

Positions

-1982-1990 INRA (National institute for agronomical research) laboratory in Lille: study of the microbiology of the methanogenic fermentation.
-1990 2007 CNRS-CERMAV in Grenoble : development of microbiological processes for the synthesis of poly and oligosaccharides

Field of expertise

Microbial physiology
Fermentation technology
Glycobiology

List of Publications

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49 Fierfort N, Samain E. (2008), Genetic engineering of Escherichia coli for the economical production of sialylated oligosaccharides. J. Biotechnol. 134, 261-265

Book chapters

R. GEREMIA & E. SAMAIN Production of heterologous oligosaccharides by recombinant bacteria (recombinant oligosaccharides) Oligosaccharides in Chemistry and biology : A Comprehensive Handbook , B. Ernst, G. Hart, P. Sinay, Eds., Wiley-VCH, 2000, 845-860

E. SAMAIN Production of oligosaccharides in microbes Comprehensive Glycoscience, 1.2 : Synthesis of Carbohydrates, éd. J.P. Kamerling, Elsevier, 0444519672 , 2007, 923-947

Patents

E SAMAIN
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E. SAMAIN, M. RANDRIANTSOA & S. DROUILLARD
Production of globoside oligosaccharides using metabolically engineered microorganisms
WO2007023348,

E. SAMAIN Efficient production of sialooligosaccharides using metabolically engineered Escherichia coli US2007020736

E. SAMAIN & B. PRIEM
Method for producing oligopolysaccharides WO0104341

SAMAIN ERIC (FR); DEBEIRE PHILIPPE (FR); DEBEIRE-GOSSELIN MICHELE (FR); TOUZEL JEAN-PIERRE (FR)
Xylanase, xylanase-producing bacillus and application thereof. WO9213942

International Invited Lectures

Samain E., Priem B., Antoine T., Dumon C.. Large scale in vivo synthesis of oligosaccharides by metabolically engineered *Escherichia coli* strains. Annual Conference of the Society for Glycobiology. Boston 9-12 novembre 2002

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Samain E Synthesis of complex oligosaccharides using metabolically engineered bacteria. 3rd Annual Carbohydrate Symposiumof the Alberta Ingenuity Centre for Carbohydrate Science 4-5 may 2007

Samain E. Synthesis of complex oligosaccharides using metabolically engineered bacteria. 54eme Symposium of the Alfred Benzon foundation (2007) Glycosylation opportunities in drug development.

EXHIBIT B

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23 April 2008

Dr. Eric Samain
CERMAV, BP53
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Dear Dr. Samain,

Thank you for your recent inquiry regarding your manuscript in the *Glycoconjugate Journal*,

> "The living factory: in vivo production of N-acetyllactosamine containing carbohydrates in E. coli." *Glycoconj.* 6(3):205-12.

The manuscript was accepted for publication on 31 March 1999 and appeared in the journal's March 1999 issue. Its actual print publication date, however, was 24 September 1999. It appeared online on SpringerLink on 28 October 2004.

Sincerely,

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EXHIBIT C

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Genetic engineering of *Escherichia coli* for the economical production of sialylated oligosaccharides

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ABSTRACT

We have previously described a microbiological process for the conversion of lactose into 3′sialyllactose and other ganglioside sugars by living *Escherichia coli* cells expressing the appropriate recombinant glycosyltransferase genes. In this system the activated sialic acid donor (CMP-Neu5Ac) was generated from exogenous sialic acid, which was transported into the cells by the permease NanT. Since sialic acid is an expensive compound, a more economical process has now been developed by genetically engineering *E. coli* K12 to be capable of generating CMP-Neu5Ac using its own internal metabolism. Mutant strains devoid of Neu5Ac aldolase and of ManNAc kinase were shown to efficiently produce 3′sialyllactose by coexpressing the α-2,3-sialyltransferase gene from *Neisseria meningitidis* with the *neuC*, *neuB* and *neuA* *Campylobacter jejuni* genes encoding *N*-acetylglucosamine-6-phosphate-epimerase, sialic acid synthase and CMP-Neu5Ac synthetase, respectively. A sialyllactose concentration of 25 g l^{-1} was obtained in long-term high cell density culture with a continuous lactose feed. This high concentration and low cost of fermentation medium should make possible to use sialylated oligosaccharides in new fields such as the food industry.

1. Introduction

N-Acetylneuraminic acid (Neu5Ac) is frequently found as a terminal sugar in cell surface complex carbohydrates and plays a major role in many biological processes such as cellular adhesion and the binding of toxins and viruses (Varki, 1993). In particular Neu5Ac is a major component of the carbohydrate portion of gangliosides, which are notably abundant in brain tissues and are involved in several pathologies (Zhang and Kiechle, 2004). Free sialylated oligosaccharides are found at high concentrations in human milk and are known to have both anti-infective and immunostimulating properties (Boehm and Stahl, 2007). They are also believed to increase the brain ganglioside and glycoprotein sialic acid concentration and favor the brain maturation of breastfed infants by serving as an exogenous source of sialic acid (Wang et al., 2003).

Due to their important biological functions, sialylated structures have attracted considerable interest and many methods have been developed for the synthesis of sialylated oligosaccharides. Since chemical synthesis are not practical because of the multiple protection and deprotection steps involved, great effort has been put into enzymatic and biotechnological methods. The development of efficient systems for the enzymatic synthesis of sialylated oligosaccharides has been possible through the identification of bacterial sialyltransferase genes which are well expressed in *Escherichia coli* and the design of multiple enzymatic systems for the synthesis of CMP-Neu5Ac (Gilbert et al., 1998). It was later shown that the cost of synthesis could be significantly reduced by using permeabilized (Endo et al., 2000) or living (Priem et al., 2002) whole *E. coli* cells. In the latter approach, lactose, which was used as exogenous acceptor, was internalized by the LacY permease and was sialylated by recombinant glycosyltransferase, using CMP-Neu5Ac, which was constantly regenerated by the enzymatic machinery of the living cells. Since the only *E. coli* strains that naturally produce CMP-Neu5Ac are pathogenic strains that cannot be used in biotechnological processes, a pathway for the synthesis of CMP-Neu5Ac had to be imported into *E. coli* strain K12 derivatives used for the production of sialylated oligosaccharides. Taking advantage of the fact that *E. coli* is able to catabolize Neu5Ac and possesses a sialic acid permease, an anabolic pathway for the synthesis of CMP-NeuAc from exogenous Neu5Ac was engineered by over-expressing the *neuA* gene for CMP-Neu5Ac synthase and by disrupting the NanA aldolase, which catalyzes the conversion of Neu5Ac into ManNAc and pyruvate. This system was first used for the production of 3′sialyllactose as illustrated in Fig. 1A and was later extended to the production of the carbohydrate portion of the gangliosides GM2

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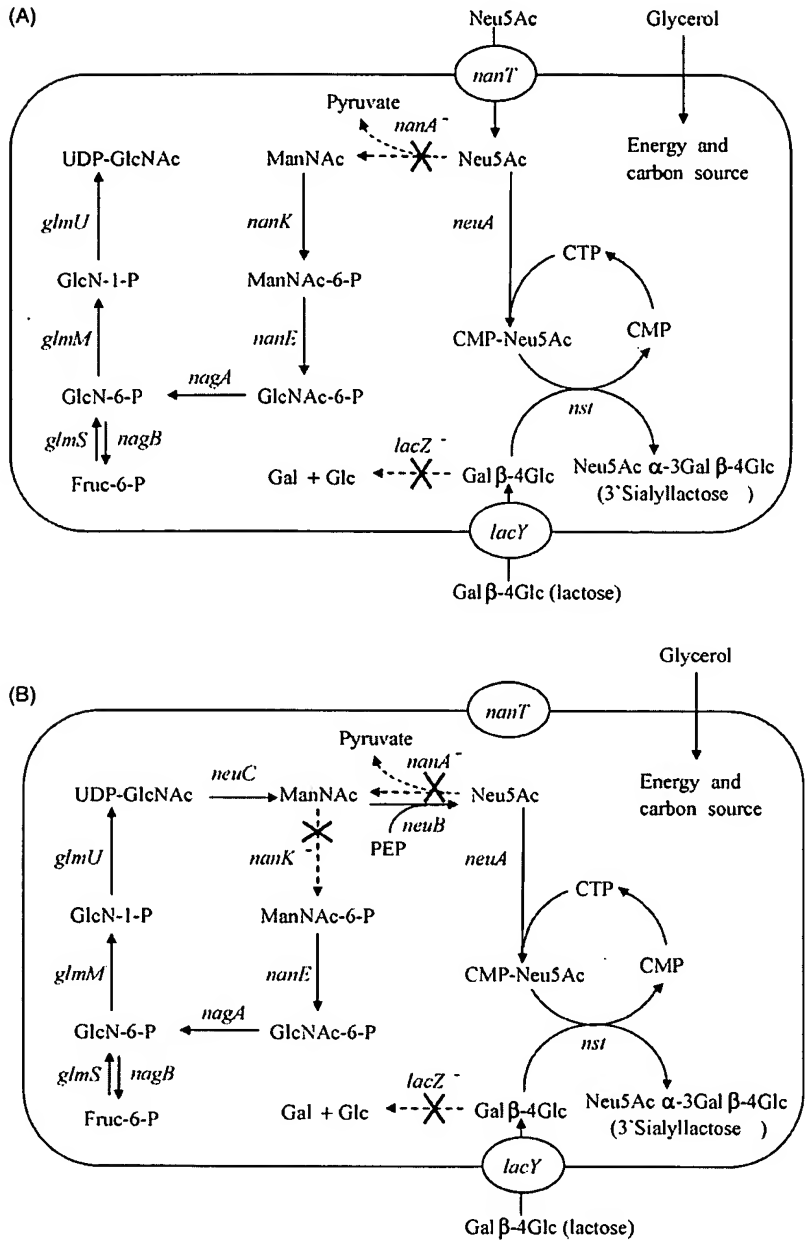

Fig. 1. Engineered metabolic pathways for the production of 3'sialyllactose with (**A**) an exogenous supply of Neu5Ac (Priem et al., 2002) or (**B**) with an endogenous synthesis of Neu5Ac from UDP-GlcNAc. Over-expressed heterologous genes are in bold. Discontinued arrows represent the enzymatic activities that have been eliminated.

and GM1 by additionally expressing the appropriate glycosyltransferase genes (Antoine et al., 2003). Polysialylated oligosaccharides (GD3 and GT3 sugars) were also produced by this method, using the *Campylobacter cstII* gene that encodes a bifunctional α-2,3- and α-2,8-sialyltransferase (Antoine et al., 2005).

Sialic acid, used as precursor for the synthesis of sialylated oligosaccharide, can be purified from natural sources such as milk and egg yolk (Koketsu et al., 1992), but the yields are low and the procedure is not suitable for large-scale production. Sialic acid is thus generally prepared by enzymatic synthesis by the sialic acid aldolase, using *N*-acetylmannosamine (ManNAc) and pyruvate as substrates. To reduce the cost, ManNAc can be prepared by chemical or enzymatic epimerization of *N*-acetylglucosamine, which is a cheaper substrate than ManNAc (Lee et al., 2004; Maru et al., 1998). In spite of these improvements, sialic acid is still relatively expensive and its cost can hamper the development of applications that require large amounts of sialylated oligosaccharides. We have thus investigated the possibility of producing sialylated oligosaccharides without an exogenous supply of Neu5Ac. In both *E. coli* K1 and *N. meningitidis*, Neu5Ac is synthesized in two enzymatic steps from UDP-GlcNAc. The first step, which is the conversion of UDP-GlcNAc into MAnNAc via a 2-acetamidoglucal intermediate (Vann et al., 2004), is catalyzed by the UDP-GlcNAc 2 epimerase encoded by the *neuC* gene. The second step is the condensation of ManNAc and phosphoenolpyruvate into Neu5Ac and is catalyzed by the sialic acid synthase encoded by the *neuB* gene (Vann et al., 1997).

In this paper we demonstrate that sialylated oligosaccharides can be economically produced by bacterial fermentation by showing that *E. coli* K12, co-expressing the three *nanABC* genes with the α-2,3-sialyltransferase genes can efficiently produce 3'sialyllactose, providing that the ManNAc kinase and Neu5Ac aldolase activities have been eliminated.

2. Materials and methods

2.1. Plasmid and strain constructions

All mutants were constructed from strain DC (Dumon et al., 2005) which was a strain DH1 derivative carrying *lacZ* and *lacA* null mutations (Table 1). To allow DNA recombination for the gene inactivation procedures, the RecA$^+$ phenotype was transiently conferred to strain DC and its derivatives as previously described (Cottaz and Samain, 2005).

Strain AZL was constructed from strain DC by inactivating *nanA* using the suicide plasmid pMAK705 (Hamilton et al., 1989), as previously described (Antoine et al., 2003).

To construct strain ZLKA from strain DC, the *nanKETA* genes were disrupted by removing a 3.339-kb segment in the chromosomal DNA using the previously described one-step procedure that employs PCR primers to provide the homology to the targeted sequence (Datsenko and Wanner, 2000). The sequence of the upstream primer was 5′GCAATTATTGATTCGGCGGATGGTTTGCCGATGGTGGTGTAGGCTGGAGCTGCTTC and the sequence of the downstream primer was: 5′CTCGTCACCCTGCCCGGCGCGCGTGAAAATAGTTTTCGCATATGAATATCCTCCTTAG.

The same procedure was used to inactivate the *nanK* gene in strain AZL to obtain strain AZK, except that the size of the deleted fragment was 0.537 kb and the sequence of the upstream primer was 5′CACTGGCGATTGATATCGGCGGTACTAAACTTGCCGCCGTGTAGGCTGGAGCTGCTTC.

The *neuBCA* genes were cloned by PCR using Pfu turbo DNA polymerase. A 2.995 DNA fragment containing the contiguous sequences of the three genes was amplified using the genomic DNA of *Campylobacter jejuni* strain ATCC 43438 as a template. A KpnI site was added to the left primer (5′GGTACCTAAGGAGGAAAATAAATGAAAGAAATAAAAATACAA) and a XhoI site was added to the right primer (5′CTCGAGTTAAGTCTCTAATCGATTGTTTTCCAATG). The amplified fragment was first cloned into the pCR4Blunt-TOPO vector (Invitrogen) and then sub-cloned into the KpnI and XhoI sites of the pBBR1-MCS3 vector to form pBBR3-SS.

2.2. High cell density culture

Cultures were carried out in 2-l reactors containing 1 l of mineral culture medium, as previously described (Priem et al., 2002). The temperature was maintained at 34 °C and the pH was regulated to 6.8 with 14% NH_4OH. The high cell density culture consisted of three phases: an exponential growth phase, which started with the inoculation of the fermenter and lasted until exhaustion of the carbon substrate (glycerol 17.5 g l^{-1}), a 5-h fed-batch with a high glycerol feeding rate of 4.5 g l^{-1} h^{-1} and a 25-h fed-batch phase with a lower glycerol feeding rate of 2.7 g l^{-1} h^{-1}. Both the lactose (7.5 g l^{-1}) and inducer (IPTG 50 mg) were added at the end of the exponential phase. In the experiment with a continuous supply of lactose, lactose was added at a concentration of 2 g l^{-1} and was then continuously fed at a rate of 0.52 g l^{-1} h^{-1} for 5 h, followed by a rate of 0.3 g l^{-1} h^{-1} for 66 h.

2.3. Quantification of oligosaccharides

Culture samples (1 ml) were centrifuged in microfuge tubes (2 min, 12,000 × *g*) just after collection. The supernatants were saved for the quantitation of extracellular oligosaccharides. The pellets were re-suspended in distilled water (1 ml), boiled for 20 min, and centrifuged (2 min, 12,000 × *g*). The second supernatant was kept for quantitation of the intracellular oligosaccharides. TLC plate analysis was carried out on silica gels and the oligosaccharides were eluted with butanol/acetic acid/water (2:1:1 two runs). Sugars were detected by dipping the plate in orcinol sulfuric reagent and heating.

2.4. Purification of sialyllactose

Sialyllactose was purified from the culture of strain DC7, which had been continuously fed with lactose. At the end of the culture, the extracellular fraction was separated from the cells by centrifugation. The pH of the extracellular fraction was lowered to 3.0 by the addition of a strong cation exchanger resin (Amberlite IR120 H$^+$ form). This resulted in the precipitation of proteins, which were removed by centrifugation. The pH of the clear supernatant was then adjusted to 6.0 by the addition of a weak anion exchanger (Dowex 66 free base form) and half of the supernatant was then loaded on a Dowex 1 (HCO_3 form) column (5 cm × 20 cm). Sialyllactose was retained by Dowex 1 resin and, after washing with distilled water, was eluted with a 0–500 mM continuous $NaHCO_3$ gradient. The same procedure was repeated with the other half of the supernatant. Eluted fractions containing sialyllactose were pooled and the $NaHCO_3$ was removed by treating with Amberlite IR120 (H$^+$ form) until pH 3.0 was reached. The pH was then adjusted to 6.0 with NaOH and the sialyllactose was freeze-dried.

For purification of the intracellular fraction, the cells were permeabilized by heating (100 °C, 45 min) and re-suspended in the same volume as the initial culture medium.

Oligosaccharides freely diffused outside the cells and were recovered in the supernatant after centrifugation. The purification of sialyllactose was then carried out using the same protocol as for the extracellular fraction. From a 1-l culture of strain DC7, the yield of purified sialyllactose was 9 g from the extracellular fraction and 6 g from the intracellular fraction. Identification of the purified product as sialyllactose was confirmed by mass spectrometry analysis.

3. Results

3.1. Production of sialyllactose by E. coli mutants expressing neuABC

Production of 3′sialyllactose was chosen as an example to demonstrate the feasibility of the microbial production of sialylated oligosaccharide without an exogenous supply of Neu5Ac. The strategy for the production of sialyllactose is described in Fig. 1B. Since the production of Neu5Ac as intermediate during the synthesis of CMP-Neu5Ac is likely to induce the sialic acid catabolism pathway and create futile cycles that could reduce the capacity of CMP-NeuAc biosynthesis, the production of sialyllactose was investigated in mutants differently affected in the catabolism of Neu5Ac. The *nanA* disruption was expected to prevent the creation of a first futile cycle, which could result from the combined activity of the sialic acid synthase NeuB with the sialic acid aldolase. Similarly the *nanK* disruption should prevent the formation of a second futile cycle, which could result from the combined action of UDP-GlcNAc 2 epimerase NeuC with the four enzymes NanK, NanE, NagA, GlmM and GlmU that catalyze the formation of UDP-GlcNAc from ManNAc. Since the four *nanKTEA* are clustered, strain ZLKA, which had the double *nanK nanA* mutation, was simply constructed by deleting the entire sialic acid gene cluster. In the hypothesis that Neu5Ac could transiently accumulate and leak out of the cells, the *nanT* deletion in strain ZLKA would prevent its re-entry in the cells and result in the loss of Neu5Ac. To check this hypothesis, the double *nanK nanA* mutant strain AZK, which had a functional sialic acid permease, was constructed by deleting only the *nanK* gene in the *nanA* mutant strain AZL. The three mutant strains AZL, AZK and ZLKA and the control strain DC were co-transformed with the plasmid

Table 1
Genes, plasmids and *Escherichia coli* strains used in this study

	Description	Reference or source
Genes		
nst	α-3 NeuAc transferase from *N. meningitidis* L3 strain MC58	U60660
neuA	CMP-Neu5Ac synthetase from *C. jejuni* ATCC 43438	AF400048
neuB	Sialic acid synthase from *C. jejuni* ATCC 43438	AF400048
neuC	GlcNAc-6-phosphate 2 epimerase from *C. jejuni* ATCC 43438	AF400048
Plasmids		
pBS-nst	pBluescript II SK derivative carrying *nst* (previously called NST-01)	Priem et al. (2002)
pBBR1MCS-3	Cloning vector, Tc^r, P_{lac} promoter, low copy number,	Kovach et al. (1995)
pBBR3-SS	pBBR1MCS-3 derivative carrying *neuABC*	This study
Strains		
DC	DH1 *lacZ lacA*	Dumon et al. (2005)
ZLKA	DC *nanKETA*	This study
AZL	DC *nanA*	This study
AZK	AZL *nanK*	This study
AW1	AZL (pBS-nst, pBBR3-SS)	This study
DC7	ZLKA (pBS-nst, pBBR3-SS)	This study
DC0	ZLKA (pBBR3-SS)	This study
AZK1	AZK (pBS-nst, pBBR3-SS)	This study

pBS-nst that contained the α-2,3-sialyltransferase gene and with the plasmid pBBR3-SS containing the *neuABC* genes from *C. jejuni* ATCC 43438. The transformant were cultivated at high cell density with glycerol as carbon and energy source in the presence of lactose as exogenous acceptor. The production of sialyllactose was estimated by the colorimetric quantitation of sialic acid in both the intra- and extra-cellular fractions (Table 2). The results showed that the control strain DC6 with no mutation in the sialic acid operon and the *nanA* mutant AW1, produced low amounts of sialyllactose with a similar production yield. By contrast the two mutants AZK1 and DC7 that carried the *nanK* and *nanA* mutations produced four times more sialyllactose. No Neu5Ac could be detected in the culture of strain DC7 carried out without lactose, indicating that the high level of total sialic acid corresponded to the formation of sialyllactose and that there was no Neu5Ac leakage into the extracellular medium. Therefore the fact of having a functional sialic acid permease has no effect on the sialyllactose production yield, as confirmed by similar yields obtained with both strains DC7 and AZK1.

3.2. Production of sialyllactose with a continuous lactose supply

In cultures of strains DC7 and AZK1, lactose ($7.5\,g\,l^{-1}$) was entirely consumed after 30 h of incubation. Improvements in sialyllactose production by extending the culture period thus require the supply of additional lactose. To prevent a possible limitation in long-term culture, lactose was added in a continuous manner. TLC analysis showed that sialyllactose (compound **2**, Fig. 2) was continuously produced until the end of culture and that sialyllactose production was not limited by the supply of lactose (compound **1**), which could always be detected in the intracellular fraction throughout the culture. Colorimetric quantification of sialic acid

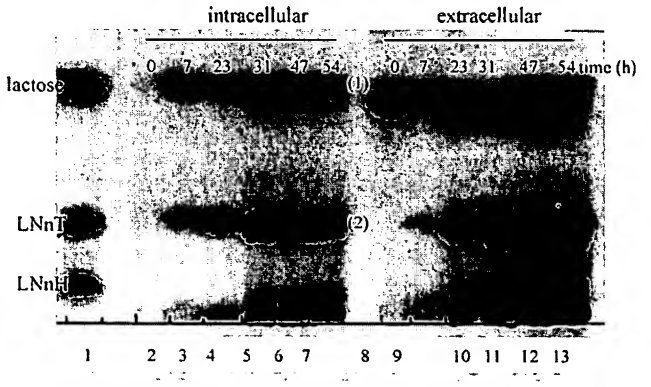

Fig. 2. TLC analysis of intracellular and extracellular fractions of the high cell density culture of strain DC7, with a continuous lactose feed. Lane 1: standard solution (2 mg ml^{-1} each) of lactose, lacto-*N*-neotetraose (LNnT, Galβ-4GlcNAcβ-3Galβ-4Glc), lacto-*N*-neohexaose (LNnH, Galβ-4GlcNAcβ-3Galβ-4GlcNAcβ-3Galβ-4Glc). Lanes 2–7: intracellular fractions withdrawn 0, 7, 23, 31, 47 and 54 h after the start of the lactose feeding. Lanes 8–13: extracellular fractions withdrawn 0, 7, 23, 31, 47 and 54 h after the start of the lactose feeding. Compound (**2**) was identified as sialyllactose (**2**) which has the same migration rate as the tetrasaccharide LNnT.

indicated that sialyllactose accumulated mainly in the intracellular fraction in the first part of the culture. The intracellular sialyllactose concentration then reached a plateau at around $10\,g\,l^{-1}$ and the additionally produced sialyllactose was then secreted into the extracellular medium where it accumulated at a final concentration of $15.5\,g\,l^{-1}$ (Fig. 3) resulting in a total (intracellular plus extracellular) sialyllactose production yield of $25.5\,g\,l^{-1}$. From a 1-l culture (initial volume), the yield of purified sialyllactose was 9 g from the

Table 2
Colorimetric quantification of sialic acid in intracellular and extracellular fractions of high cell density cultures of strains genetically engineered for the production of sialyllactose

Strain	Disrupted genes	Heterologous genes expressed	Acceptor	Total sialic acid concentration ($g\,l^{-1}$)	
				Intracellular	Extracellular
DC		None	Lactose	0	0
DC6		*neuBCA nst*	Lactose	0.94	0.43
AW1	*nanA*	*neuBCA nst*	Lactose	1.13	0.27
DC7	*nanKETA*	*neuBCA nst*	Lactose	2.32	3.25
DC7	*nanKETA*	*neuBCA nst*	None	0.11	0
DC0	*nanKETA*	*neuBCA*	None	0	0
AZK1	*nanK nanA*	*neuBCA nst*	Lactose	2.16	2.93

Total sialic acid was quantitated by the diphenylamine method (Werner and Odin, 1952).

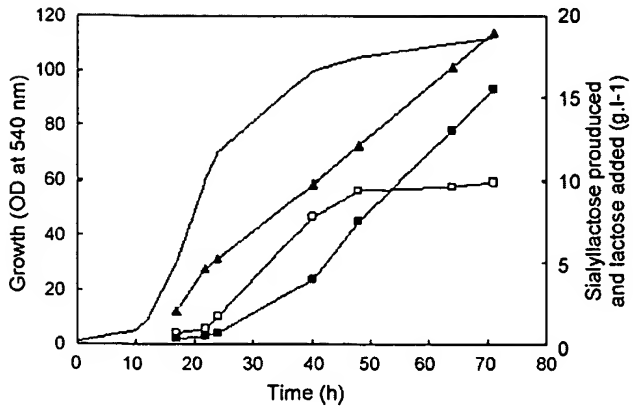

Fig. 3. Production of sialyllactose in a high cell density culture of strain DC7 with a continuous lactose feed: (▲) cumulated amount of added lactose; (□) intracellular sialyllactose; (■) extracellular sialyllactose; (–) bacterial growth.

extracellular fraction and 6 g from the intracellular fraction. Identification of the purified product as sialyllactose was confirmed by mass spectrometry analysis.

4. Discussion

These results demonstrate that the over-expression of the *neuABC* genes confers on *E. coli* K12 the CMP-Neu5Ac biosynthetic capacity to efficiently produce sialylated oligosaccharides, such as 3′sialyllactose. The knockout of the *nanK* and *nanA* genes improves considerably the sialylation efficiency of the system by preventing ManNAc and Neu5Ac from being diverted from the biosynthesis of the CMP-Neu5Ac pathway and by avoiding the formation of energetically deleterious futile cycles.

In animals the CMP-Neu5Ac biosynthesis flux is regulated by the activity of UDP-GlcNAc 2-epimerase, which has been shown to be feedback inhibited by CMP-Neu5Ac (Kornfeld et al., 1964). In sialuria, a sialic acid storage disorder, free sialic acid accumulates due to a defect in the regulation of UDP-GlcNAc 2-epimerase by CMP-Neu5Ac. The mechanism for regulating CMP-NeuAc biosynthesis in bacteria has not been determined. However, bacterial UDP-GlcNAc 2-epimerases show high sequence similarities with their animal counterparts and it is likely that a similar mechanism of feedback inhibition by CMP-Neu5Ac also exists in bacteria. This would explain the absence of Neu5Ac accumulation in cultures of strain DC0 that express *neuABC* without expressing sialyltransferase and in cultures of strain DC7 without lactose. This regulation mechanism is technologically very interesting because it prevents the possibility of Neu5Ac leakage in the extracellular medium.

The sialyllactose production yield of 25.5 g l^{-1} obtained in long-term cultures of strain DC7 is 10 times higher than the yield of 2.6 g l^{-1} previously reported with our initial system based on an exogenous supply of Neu5Ac (Priem et al., 2002). The cost of sialic acid was indeed the major constraint for optimizing the microbial production of sialyllactose, which can now be favorably envisaged due to the low cost of the different culture media ingredients and the high volumetric productivity. Industrial implementation of this system should lead to the production of sialyllactose at a cost that would allow its use in various fields such as in the food industry. However, the fact that the heterologous genes used in this system are derived from pathogenic bacteria could be negatively regarded by consumers and it would be therefore interesting to find equivalent genes from non-pathogenic organisms.

This economical system of sialyllactose production can obviously be extended to the production of a large number of sialylated oligosaccharides of biological interest. The system previously developed for the microbial production of GM1, GM2, and GD3 sugars can be adapted accordingly to avoid the supply of Neu5Ac. Other sialylated oligosaccharides, such as those found in human milk, would certainly represent an interesting development for our new sialylation system.

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